

EDNA MONITORING OF A RARE SALAMANDER: THE USE OF EDNA IN DETECTION OF EURYCEA JUNALUSKA IN THE GREAT SMOKY MOUNTAINS NATIONAL PARK, USA

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Abstract.—The Junaluska Salamander (*Eurycea junaluska*) is a small, semiaquatic plethodontid salamander with a restricted range, much of which is encompassed within the Great Smoky Mountains National Park (GSMNP). *Eurycea junaluska* was first described in 1976 and is known to exist in only five counties in a small area of Tennessee and North Carolina. Environmental DNA (eDNA) is an emerging tool that promises to greatly increase the ease, efficacy, and scope of ecological studies. The objectives of this study were to develop and validate a species-specific assay that detects *E. junaluska* and to utilize this assay to reassess the distribution of *E. junaluska* within the boundaries of the GSMNP. We sequenced a portion of the mitochondrial gene cytochrome b (cytb) from *E. junaluska* and sympatric salamander species, designed a qPCR assay for *E. junaluska* detection, and confirmed the specificity of this assay *in silico* and *in vitro* against all other salamander species found in the GSMNP and two additional *Eurycea* species. We collected 50 water samples from 30 of 46 U.S. Geological Survey hierarchical hydrologic unit code 12-digit sub-watersheds found within the GSMNP, including all major watersheds. We collected water samples in first to sixth order streams, and we used the developed qPCR assay to detect *E. junaluska* eDNA in the extracted water samples. Target DNA was detected at four sites, all in large, low-elevation, relatively low-gradient streams, each with extensive historic observation records of *E. junaluska*. Occupancy modeling indicated a low probability of false positive, but a somewhat higher probability of false negative, errors as well as the significance of covariates influencing the probability of *E. junaluska* detection including elevation, Strahler stream order, and location of the site within the GSMNP.

Key Words.— environmental DNA; Junaluska Salamander; single-species eDNA assay, eDNA occupancy modeling

INTRODUCTION

Following its initial use in vertebrate studies by Ficetola et al. (2008), environmental DNA (eDNA) has become widely used as a method for field studies and surveys, capable of determining organism presence (Goldberg et al. 2011; Kaganer et al. 2022; Moss et al. 2022) and perhaps abundance (Everts et al. 2021, 2022; Breton et al. 2022). With technological advances promoting the development of assays, primers, and other molecular tools, eDNA has greatly expanded the toolset available for conducting ecological studies. eDNA studies using DNA extracted from a variety of substrates, such as water, soil, and air, have now been completed (Littlefair et al. 2023; Vasar et al. 2023), although studies using water-extracted DNA are most prevalent (Duarte et al. 2023). The recent utilization of metabarcoding in eDNA studies allows the detection of hundreds of species simultaneously (Lodge 2022; Mu et al. 2024;

Vanderpool et al. 2024), but single-species assays may offer lower detection limits (Harper et al. 2018; Bylemans et al. 2019; Wood et al. 2019; Banks et al. 2021; Moss et al. 2022), lower cost (Langlois et al. 2020), and are recommended over metabarcoding when study objectives are the detection of one or a small number of species (Goldberg et al. 2016; Wood et al. 2019). One of the areas in which single-species DNA assays remain most successful is in the detection of species so rare that traditional survey approaches are either ineffective, problematic, or prohibitive because of labor and cost considerations (Wilcox et al. 2013; Duarte et al. 2023).

The Junaluska Salamander (*Eurycea junaluska*; Fig. 1), is a small, semi-aquatic plethodontid salamander with a restricted range, much of which is encompassed within the Great Smoky Mountains National Park (GSMNP) of the U.S. (Green et al. 2014). *Eurycea junaluska* was first described in 1976 and at that time was only known to exist in three creeks in Graham



FIGURE 1. A Junaluska Salamander (*Eurycea junaluska*) metamorph from eastern Tennessee, USA. (Photographed by Todd Pierson).

County, North Carolina, USA (Sever et al. 1976). A re-examination of specimens previously collected by King (1939) and new fieldwork expanded the range to several locations on both the Tennessee, USA, and North Carolina sides of the GSMNP, as well as to adjacent Polk County, Tennessee (Bruce 1982; Sever 1983; Ryan 1998). Currently, *E. junaluska* is known to occur in only five counties in Tennessee and North Carolina in the upper Tennessee River Drainage (Ryan and Sever 2005).

The endemism of *E. junaluska* makes it vulnerable to threats that are of lesser concern to more widely distributed species (Ryan 1997). Chattin et al. (2007) report that *E. junaluska* is likely to be exceptionally susceptible to stream channel alterations, and Ryan (1998) described the persistence of *E. junaluska* as depending on access to restricted key larval habitats. Furthermore, the disjunct nature of the population means it is highly unlikely that migration would result in the recolonization of sites following local extinctions, which are possible or likely given the low abundance of individuals in isolated populations (Ryan 1997, 1998). Additionally, Chattin et al. (2007) suggest that many of the current *E. junaluska* populations may occupy sub-optimal habitat and highlight the need for further study of this species. Although a proposal to list *E. junaluska* as a federally listed Endangered Species was denied (U.S. Fish and Wildlife Service 1999), the species is currently listed in Tennessee as In Need of Management (<https://www.tn.gov/twra/wildlife/amphibians/salamanders/junaluska.html>) and as Threatened in North Carolina (North Carolina Wildlife Resource Commission 2021).

Adult *E. junaluska* are notoriously difficult to locate (Bruce 1982; Sever 1983), and larval surveys have been suggested as the most reliable indicator of population location and stability (Sever 1983;

Ryan 1997). Adult *E. junaluska* are described as predominantly aquatic and streamside (Dodd 2003) and the fully aquatic larval period is 2–3 y in length (Bruce 1982; Ryan 1998), guaranteeing that individuals are continually in contact with the water if populations are present. Additionally, larval *E. junaluska* are difficult to distinguish from sympatric Blue-lined Salamander (*Eurycea wilderae*) larvae (Ryan 1997), perhaps the most abundant salamander larvae in the park (Dodd 2004). Several researchers have concluded that morphologically distinguishing these two species is impossible for total body lengths under 20 mm (Bruce 1982; Sever 1983). A survey for salamanders using leaf litter bags in GSMNP streams reported capture of 381 indistinguishable *E. junaluska*/*E. wilderae* larvae (< 18 mm), constituting 55% of all larvae captured in the study (Waldron et al. 2003). Development of a species-specific eDNA assay has the potential to enable differentiation between these two closely related species at any life stage through water sample analysis or by testing specimens individually through swab or tissue samples. For these reasons, *E. junaluska* represents an ideal candidate for eDNA monitoring.

Numerous studies report successful detection of salamander species in lotic systems of differing size. Katano et al. (2017) reported detection of the Japanese Clawed Salamander (*Onychodactylus japonicus*) in headwater tributaries of the Ibo River in Hyogo, Japan. Plante et al. (2021) reported congruence between traditional surveys and eDNA detection of three salamander species, including the Northern Two-Lined Salamander (*Eurycea bislineata*) in 24 headwater streams in eastern Canada (Quebec). Bell et al. (2022) reported successful detection of the Southern Two-Lined Salamander (*Eurycea cirrigera*) and the Northern Dusky Salamander (*Desmognathus fuscus*) in low-order headwater streams in the southern Appalachians in eastern Kentucky, USA. Other studies have successfully detected salamanders in larger lotic systems. Pilliod et al. (2013) reported successful detection of Idaho Giant Salamanders (*Dicamptodon aterrimus*) in first to third order streams in Idaho, USA. Santas et al. (2013) reported detection of Eastern Hellbenders (*Cryptobranchus alleganiensis*) in high-order, relatively low-elevation Kentucky streams with mean collection site stream widths of 13.2 m. Wineland (2018) reported detection of Eastern Hellbenders in 19/22 sites in fourth order or larger West Virginia, USA, streams. Our objectives were to design and validate a single-species eDNA assay for use in the detection of *E.*

junaluska and to complete an eDNA survey for *E. junaluska* in the GSMNP.

MATERIALS AND METHODS

Methodological overview.—We first sequenced a portion of cytochrome b from our target species (*E. junaluska*) as well as from closely related, non-target, sympatric species collected within or just outside our study area. We then developed an eDNA assay and tested it using computer simulations (*in silico*) and extensively *in vitro* using genomic DNA, plasmid clones, and/or cytochrome b gBlocks from the target and all sympatric *Eurycea* species. We completed standard curves using cytochrome b (cytb) gBlocks of our target species and determined LOD and LOQ utilizing a modeling framework. We then collected single water samples from 50 sites within the GSMNP, including a field blank for every 10 samples collected. We field-filtered water samples and extracted DNA in the laboratory. Following inhibition testing, we ran qPCR analysis in triplicate and included both positive and negative controls. We Sanger-sequenced one amplicon from every site yielding a positive *E. junaluska* detection to confirm species identity. Finally, we used occupancy modeling to analyze the probability of organism presence, the probability of eDNA collection in a sample from a site occupied by the target species, and the probability of qPCR amplification in a sample containing target organism DNA, as well as the potential significance of covariates.

Sequencing of target and non-target species.—To assure the primers and probe designed for eDNA detection recognized the target species and not closely related non-target species, we amplified portions of cytb from our target species and closely related sympatric species. Currently, four *Eurycea* species exist in sympatry with *E. junaluska*: *Eurycea wilderae*, Long-tailed Salamanders (*Eurycea longicauda*), Cave Salamanders (*Eurycea lucifuga*),

and Three-lined Salamanders (*Eurycea guttolineata*). We sequenced DNA from both *E. junaluska* (one specimen) and *E. wilderae* (four specimens), the most closely related *Eurycea* species occurring (Edgington and Taylor 2019) sympatrically with *E. junaluska*. DNA was either donated as tissue or collected within the park via swabs. We extracted DNA using a DNeasy Blood and Tissue Kit (Qiagen; Venlo, Netherlands) following a modified version of the protocol of the manufacturer: we lysed tissue overnight at 56° C in Proteinase K and eluted twice (200 µl and then 100 µl, 300 µl total) to increase DNA yield. To amplify a portion of cytb from our *E. junaluska* sample, we used the published primer set MVZ15 (F) + MVZ18 (R) (Moritz et al. 1992); for our *E. wilderae* samples, we designed a novel forward primer based on published sequences, *Eurycea* cytb for. (F), and used a published reverse primer, MVZ16 (R) (Moritz et al. 1992). Each primer used is listed in Supplemental Information Table S1 with its respective sequence and is also included within the GenBank submission of its respective sequence. We Sanger-sequenced the resulting PCR products in duplicate (ACGTinc.com) and then aligned and edited each sequence prior to GenBank submission.

Assay development and testing.—We used Mega X (Version 10.2.4; Kumar et al. 2018) and Clustal W to align the two available cytb sequences of *E. junaluska* (the sequence generated in this project and the existing published sequence) and cytb sequences from the four other *Eurycea* species found in the GSMNP. We used IDT PrimerQuest to design a forward primer, a reverse primer, and a probe (Table 1) that recognize a region that is conserved between the two *E. junaluska* sequences (collected from opposite ends of the *E. junaluska* range) but that do not recognize other sympatric *Eurycea* species. We then screened these oligonucleotides (oligos) for specificity *in silico* by aligning them with sequences from the 30 available cytb sequences from non-target salamander species found within the GSMNP

TABLE 1. Quantitative PCR assay developed for the Junaluska Salamander (*Eurycea junaluska*) from the Great Smoky Mountains National Park, USA. The assay amplifies an 81-base pair (bp) region of the cytochrome b gene. T_m signifies the melting temperature of the primer.

Oligo	T_m (° C)	Length (bp)	Sequence (5'-3')
Forward primer	61.6	23	GCACTATACTGCAGATACTTCCT
Reverse primer	61.7	28	CGTACTAGTCAACCATAATTTACATCTC
Probe	65.4	22	CCGCATTCTCCTCTGTAGCTCA

(sequences for Cherokee Black-bellied Salamanders, *Desmognathus gvnigeusgwotli*, were not available). Additionally, because published cytb sequences were not available for *E. wilderae* populations found in or near the GSMNP, we obtained DNA from four *E. wilderae* specimens collected within or adjacent to the park and sequenced a region of cytb from each specimen (Supplemental Information Table S2). Accession numbers of the cytb sequences used for *in silico* alignments are found in Appendix Table. Prediction of primer specificity based solely on the number and position of mismatches can be misleading (So et al. 2020); therefore, we used a machine learning program (eDNAAssay software: Kronenberger et al. 2022) to predict assignment probabilities (into amplification or non-amplification classes) of potential sympatric, non-target species (Appendix Table and Supplemental Information Table S2). As suggested by Kronenberger et al. (2022), we included non-target species yielding an assignment probability of ≥ 0.3 during *in vitro* testing. Additionally, we included *E. lucifuga* during *in vitro* testing despite a slightly lower than threshold (0.27) assignment probability. We also included Brownback Salamanders (*Eurycea aquatica*) and *E. cirrigera* in the *in silico* analysis. The range of *E. aquatica* is allopatric but close to *E. junaluska* (Stuart et al. 2020). Although the distribution of *E. cirrigera* is currently allopatric, taxonomic revisions may result in a small zone of sympatry with *E. junaluska* (David Beamer, pers. comm.).

We completed *in vitro* assay specificity testing through the acquisition of known copy number dilutions through plasmid cloning (seven specimens, including *E. junaluska*) or gBlocks (*E. guttolineata*, for which were unable to obtain tissue). Although non-linearized plasmids are commonly used in eDNA study standard curves (Takeuchi et al. 2019; Takeshita et al. 2020; Saito and Doi 2021), the supercoiled nature of plasmid DNA has been demonstrated to inhibit target DNA amplification and can lead to 2.6- to 8-fold differences in copy number estimations relative to linear DNA (Hou et al. 2010; Lin et al. 2011). For *in vitro* testing, we used DNA concentrations approximately 1.2-fold (10,000 copies per reaction) and 13-fold (100,000 copies per reaction) higher than the highest salamander eDNA concentrations observed in the environment of which we are aware in the literature (Plante et al. 2021). We conducted plasmid cloning by first amplifying a portion of cytb for each specimen using a combination of published primers and primers novel

to this study (Supplemental Information Table S1). We ligated PCR products to pGEM-T Easy plasmid vectors (Promega.com) and transformed plasmids into DH5 α competent *E. coli* cells. We grew cells on tryptic soy agar plates containing ampicillin and X-gal and then selected white colonies to grow in tryptic soy broth. The plasmid DNA of each culture was subsequently purified using QIA-prep miniprep spin columns (Qiagen.com) and was sequenced by ACGT, Inc. (ACGTinc.com) to confirm sequence identity. We designed double-stranded synthetic DNA gBlocks from IDT which consisted of a 499 bp region, including our assay binding sites, of an *E. guttolineata* specimen from Berkley County, South Carolina, USA (GenBank Accession # JQ920625.1; Supplemental Information Table S3). Our gBlocks sequence is 98.6% similar to the only other *E. guttolineata* cytb sequence available in GenBank which includes our assay binding sites, and *E. guttolineata* is known to be genetically homogenous throughout large sections of its range (David Beamer, pers. comm.). Sequence and locality information for each of the sequences included in the *in vitro* testing is provided in Supplemental Information Table S2.

We initially tested the *E. junaluska* forward and reverse primers using end-point PCR reactions, including a gradient reaction to determine optimal annealing temperature (Supplemental Information Fig. S1) and specificity tests with seven sympatric salamander species (Supplemental Information Fig. S2). Following the addition of the probe, we conducted specificity tests by running qPCR reactions with eight templates: an *E. junaluska* plasmid clone, the *E. junaluska* gBlocks, and DNA from six non-target sympatric species (five plasmid clones and one gBlocks). Non-target species we tested included all sympatric *Eurycea* species (including two specimens of *E. wilderae*) as well as *E. cirrigera*. We ran reactions with both 10,000 copies of the template per reaction (in duplicate) and 100,000 copies per reaction (in triplicate). Each 10.0 μ l reaction contained the following: TaqManTM EMM 2.0 (5.0 μ l), nuclease-free water (3.5 μ l), plasmid or gBlocks DNA (1.0 μ l), and our assay (0.5 μ l). Final reaction concentrations were 500 nM (F + R primer) and 250 nM (probe). These final primer and probe concentrations were based on concentrations used in previous studies (Bayer et al. 2019; Kageyama et al. 2022; Jones et al. 2024), including similar studies using eDNA to detect salamanders (Bell et al. 2022; Brammell et al. 2023). Although these primer and probe parameters were functional in this study, primer/probe concentration

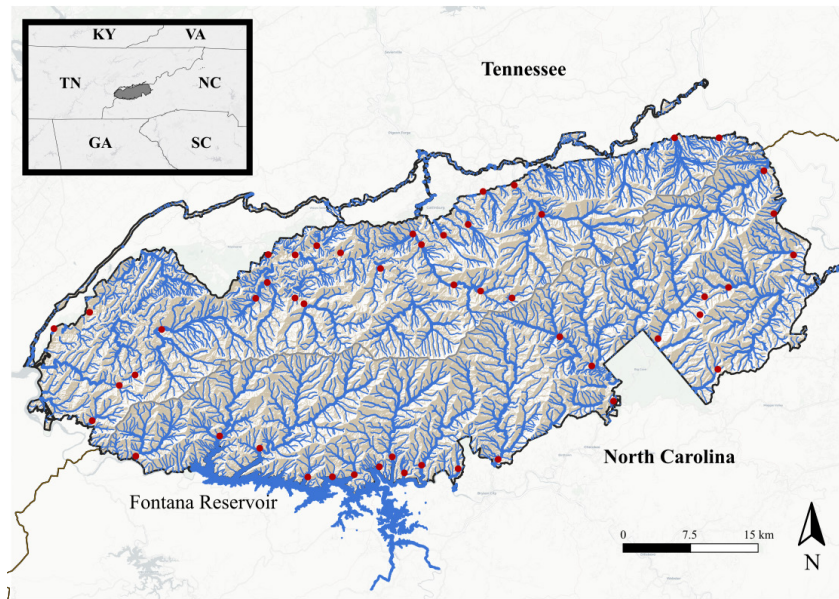


FIGURE 2. Aquatic eDNA sample collection sites in the Great Smoky Mountains National Park, USA. All water samples were collected 14–21 July 2023.

optimization could improve assay performance. Thermocycler conditions were as follows: 95° C for 10 min followed by 55 cycles of 95° C for 15 s and 60° C for one min. Reaction components and thermocycler conditions were identical to those used in the sample analysis and standard curve. We completed additional qPCR specificity testing using genomic DNA dilutions (1.0–0.001 µg/ml) of three *E. wilderae* specimens, one *E. lucifuga* specimen, and one *E. longicauda* specimen (Table 2).

Standard curves.—We used synthetic DNA to assess assay sensitivity to enable standardization and interlaboratory comparison (Hobbs et al. 2019; Klymus et al. 2020). We used a 500 bp double-stranded synthetic gBlocks (IDT) containing the 81 bp region targeted by our primers (Supplemental Information Table S3) to complete standard curves. The *E. junaluska* gBlocks were identical to the *E. junaluska* specimen collected in Blount County, Tennessee (GenBank Accession # KF562550.1), although as previously mentioned, the primer and probe binding sites were conserved between the two *E. junaluska* specimen sequences available in GenBank. We resuspended the gBlocks in IDTE, pH 8.0 (IDT), confirmed its concentration with a Qubit™ 3.0 fluorometer, and serially diluted it in nuclease-free water to produce a range of ten synthetic DNA concentrations. We ran 2 µl of each dilution in qPCR reactions with five technical replicates. The final range

tested per reaction was therefore 2.0×10^8 to 0.2 copies per reaction. Each 20.0 µl reaction contained the following: TaqMan™ EMM 2.0 (10.0 µl), nuclease-free water (7.0 µl), resuspended gBlocks (2.0 µl), and our assay (1.0 µl). Thermocycler conditions were as follows: 95° C for 10 min followed by 55 cycles of 95° C for 15 s and 60° C for one min. We used the curve-fitting model approach described by Klymus et al. (2020) to determine the limits of detection (LOD) and limits of quantification (LOQ) using their R script (Merkes, C.M., K.E. Klymus, M.J. Allison, C. Goldberg, C.C. Helbing, M.E. Hunter, C.A. Jackson, R.F. Lance, A.M. Mangan, E.M. Monroe, et al. 2019. Generic qPCR Limit of Detection (LOD) / Limit of Quantification (LOQ) calculator. R Script. cmerkes. Available from https://github.com/cmerkes/qPCR_LOD_Calc [Accessed 18 March 2025]) in RStudio (R Core Team 2023). We used a 95% probability of detection for LOD calculation and a threshold of 35% coefficient of variation to determine the LOQ.

Field sampling and processing of eDNA samples.—We collected 50 water samples throughout the GSMNP between 14–21 July 2023 (Fig. 2). We collected water samples in 30 (Table 3) of the 46 U.S. Geological Survey (USGS) hierarchical hydrologic unit code (HUC) 12-digit sub-watersheds found within the GSMNP (Bates et al. 2018). *Eurycea junaluska* has been reported to be more common in higher-order streams but also present in low-order

TABLE 2. *In vitro* qPCR testing of our Junaluska Salamander (*Eurycea junaluska*) assay with three Blue Ridge Two-lined (*Eurycea wilderae*) specimens, the Long-tailed Salamander (*Eurycea longicauda*), and the Cave Salamander (*Eurycea lucifuga*). Numbers beside parentheses represent crossing threshold (Ct) values; numbers inside parentheses represent number of positive qPCR replicates. An asterisk (*) = non-published sequence. U.S. state abbreviations are GA = Georgia, KY = Kentucky, NC = North Carolina, and TN = Tennessee.

Species	Collection locality	GenBank Acc. #	DNA conc. (µg/ml)					
			1.0	0.5	0.25	0.1	0.01	0.001
<i>E. junaluska</i>	Graham Co., NC	PP277535.1	21.9 (5/5)	22.8 (1/1)	23.3 (2/2)	26.1 (2/2)	29.8 (2/2)	33.6 (2/2)
<i>E. wilderae</i>	Cocke Co., TN	PP393048.1	0/3	—	—	—	—	—
<i>E. wilderae</i>	Cocke Co., TN	PP393049.1	0/3	—	—	—	—	—
<i>E. wilderae</i>	Rabon Co., GA	PP393050.1	36.3 (4/4)	37.3 (4/4)	36.9 (3/3)	37.9 (2/2)	(0/2)	(0/2)
<i>E. lucifuga</i>	Pulaski Co., KY	*	40.0 (1/2)	(0/3)	—	—	—	—
<i>E. longicauda</i>	Pulaski Co., KY	*	(0/2)	—	—	—	—	—

lotic habitats (Dodd 2004); therefore, we attempted to distribute our sampling effort to include all stream orders. Streams that we sampled varied between first and sixth order, according to the Strahler method of stream classification (Strahler 1957). We included two seeps/temporary streams that did not appear on USGS topographical maps in the project.

We vacuum filtered one-liter water samples in the field using a hand pump and 250-ml, 47 mm Single Use Analytical Filter Funnels (Nalgene; Rochester, New York, USA) with 0.45 µm cellulose nitrate membranes. We transferred stream water from the stream to filter using a new, disposable polyethylene cup that was opened on site from its packaging and thrice rinsed with site stream water before transferring. We used forceps and scissors to manipulate filters; these were dipped in ethanol and thrice flamed before contact with new samples. We transferred filters to sterile 1.5 ml Eppendorf microcentrifuge tubes containing 1 ml ATL extraction buffer (Qiagen), a recommended method of storage (Majaneva et al. 2018), and we held them on ice until arrival in the lab (1–7 d later). Each stored filter was held in the lab at 4° C for 7–21 d until extraction. We filtered a field blank per every 10 field samples collected. Field blanks consisted of bottled distilled water filtered in the location where the last sample in the set of 10 was collected, excluding one weather-related event that forced the completion of the blank in a nearby location. We completed and processed field blanks in a manner identical to field-collected samples.

We extracted eDNA using a DNeasy Blood and Tissue Kit (Qiagen) in a manner similar to previous studies (Hinlo et al. 2017) and used a modified version of a published protocol (Goldberg et al. 2011). We cut the filters in half, and half of each filter was stored in ethanol for potential future use; none of these stored

halves were used in this study. We cut the other filter halves into 15–20 pieces and incubated them at 56° C overnight in 360 µl ATL buffer and 40 µl Proteinase K (double the volumes recommended for tissue). We conducted extractions according to the DNeasy kit protocol with the final elution performed twice (first with 200 µl and then with 100 µl of AE buffer, 300 µl total). We stored extracted DNA at 4° C until analysis.

We carried out all extractions in a laminar flow cabinet that had been sterilized with bleach and located in a room physically separated from where PCR products were handled (Taberlet et al. 1999). We had previously washed all equipment used (forceps, scissors) with a 50% bleach solution (Goldberg et al. 2016), ethanol-flamed them, and subsequently rinsed them with deionized water. We regularly cleaned laboratory surfaces on which PCR reactions were performed with a 50% bleach solution, and equipment used in the PCR laboratory did not leave the clean laboratory (Goldberg et al. 2016).

qPCR analysis.—We quantified eDNA using a StepOnePlus™ Real-Time PCR system (Life Technologies; Carlsbad, California, USA) in optical eight-well PCR strips. Each run contained tissue-extracted target species DNA (1.0 µg/ml) as a separate, non-internal positive control and included a non-template negative control (molecular-grade, nuclease-free sterile water). Each 20.0 µl reaction contained the following: TaqMan™ EMM 2.0 (10.0 µl), nuclease-free water (2.0 µl), eDNA extract (7.0 µl), and our assay (1.0 µl); final reaction concentrations were 500 nM (F + R primer) and 250 nM (probe). Primer and probe concentrations were identical to those previously described for the standard curve. Thermocycler conditions were as follows: 50° C for 2

TABLE 3. Water sample collection sites by primary drainage and U.S. Geological Survey (USGS) hierarchal hydrologic unit code (HUC) 12-digit sub-watershed. The abbreviation # samples = the number of water samples collected within each watershed, # positive = the number of water samples in which at least one qPCR replicate of three was positive for the Junaluska Salamander (*Eurycea junaluska*) DNA, and stream order(s) indicates the Strahler (1957) stream order of water samples taken within the watershed. Specific HUC watershed codes were withheld for Abrams Creek and Little River at the request of the administrators of the Great Smoky Mountains National Park, USA. An asterisk (*) = Seeps/ephemeral streams that did not appear on U.S. Geological Survey topographical maps.

Drainage	Watershed	# samples	Stream order(s)	# positive
Abrams Creek	Abrams Creek (two sampled)	5	1 st , 2 nd (2), 4 th , 5 th	2
Big Creek	Big Creek	1	4 th	
Calderwood Reservoir	Lake Cheoah-Little TN River	1	4 th	
Cataloochee River	Cataloochee Creek	1	5 th	
Cataloochee River	Palmer Creek	1	2 nd	
Chilhowee Reservoir	Chilhowee Lake-Little TN River	1	3 rd	
Cosby Creek	Cosby Creek	1	4 th	
Deep Creek	Deep Creek	1	4 th	
Dudley Creek	Dudley Creek	1	3 rd	
Eagle Creek	Eagle Creek	1	3 rd	
Fontana Reservoir	Fontana Lake-Tuckasegee River	3	1 st (2), 3 rd	
Fontana Reservoir	Forney Creek	1	4 th	
Fontana Reservoir	Hazel Creek	1	5 th	
Fontana Reservoir	Lower Fontana Lake-Little TN River	3	1 st , 3 rd (2)	
Fontana Reservoir	Noland Creek	1	4 th	
Little Pigeon	Upper Little Pigeon River	1	5 th	
Little Pigeon	Upper West Prong Little Pigeon River	5	2 nd (2), 3 rd , 4 th (2)	
Little Pigeon River	Middle Prong Little Pigeon River	1	4 th	
Little River	Little River (two sampled)	5	2 nd , 3 rd , 5 th (2), 6 th	2
Little River	West Prong Little River	4	1 st , 3 rd , 4 th , 5 th	
Oconaluftee River	Lower Oconaluftee River	1	5 th	
Oconaluftee River	Upper Oconaluftee River	2	4 th	
Pigeon River	Cold Springs Creek-Pigeon River	1	2 nd	
Pigeon River	Cripple Creek-Pigeon River	1	2 nd	
Raven Fork	Lower Raven Fork	1	1 st	
Roaring Fork	Roaring Fork	1	4 th	
West Prong Little Pigeon	Le Conte Creek	1	2 nd	
West Prong Little Pigeon	Straight Fork Raven Fork	3	0*(2), 4 th	

min, 95° C for 10 min, and 55 cycles of 95° C for 15 s and 60° C for 1 min. We ran each sample in triplicate, similar to previously published studies (Shen et al. 2013; Sansom and Sassoubre 2017; Brammell et al. 2023). We considered a sample positive if at least one qPCR replicate out of three amplified (Harper et al. 2018; Plante et al. 2021).

We completed inhibition testing for each field-extracted sample using TaqMan™ Exogenous Internal Positive Control Reagents (Applied

Biosystems). Each 20.0 µl reaction contained the following: TaqMan™ EMM 2.0 (10.0 µl), nuclease-free water (0.6 µl), eDNA extract (7.0 µl), IPC assay (2.0 µl), and IPC DNA (0.4 µl). Positive controls were included for comparison in which the extracted sample (which potentially contained inhibitors) was replaced with nuclease-free water. Thermocycler conditions were as follows: 50° C for 2 min, 95° C for 10 min, and 40 cycles of 95° C for 15 s and 60° C for 1 min. We considered wells showing no exponential

amplification or delayed amplification (a Ct value which exceeded the Ct of the positive control runs by three or more) to be inhibited (Hartman et al. 2005; Pierson et al. 2016). We treated samples that demonstrated inhibition (five) with a One-Step PCR Inhibitor Removal Kit (IRK, Zymo Research) to remove potential PCR inhibitors. Initially inhibited samples were retested post inhibition removal to determine the efficacy of the removal process.

Amplicon sequencing.—We used tailed primers (Majaneva et al. 2018) in a secondary PCR reaction to extend the length of the 81 bp amplicons obtained from field-extracted samples and sequenced them to verify amplicon identity. We sequenced one amplicon from each of the sites positive for *E. junaluska* DNA. We unidirectionally sequenced PCR products via Sanger using either the forward or reverse primer (ACGTinc.com).

Occupancy modeling.—We used an occupancy modeling approach to calculate the probability of *E. junaluska* occupancy and to evaluate potential false negative and false positive errors from sample collection in the field and from PCR work in the laboratory. We used a statistical method that accounts for false positive and false negative errors in single-species eDNA assays developed by Griffin et al. (2020) and executed it using the R package eDNAShinyApp (Diana et al. 2021). This application was developed for eDNA occupancy modeling and has been used by many eDNA studies (Buxton et al. 2022; Lyman et al. 2022; Kalogianni et al. 2023; Osathanunkul and Suwannapoom 2024). We completed all calculations in R Studio version 4.4.2 (R Core Team 2023). The probability of presence or absence of a species is set randomly as a covariate by this model, enabling determination of: (1) Probability of target species presence at a site (ψ); (2) Probability that a sample from an occupied site contains the target species DNA (true positive; θ_{11}); (3) Probability that a sample from an unoccupied site contains the target species DNA (false positive; θ_{10}); (4) Probability of PCR amplification in a sample that contains the target species DNA (true positive detection; p_{11}); and (5) Probability of PCR amplification in a sample that does not contain the target species DNA (false positive detection; p_{10}). We calculated false negative probabilities in the sample collection and qPCR replicate as $1-\theta_{11}$ and $1-p_{11}$, respectively. We included the following covariates in the model as predictors of target species presence: elevation from

which the sample was taken (meters above sea level), Strahler stream order (Strahler 1957), and whether the eDNA sample was collected from the east or west side of the park. Additionally, we included the historic collection data of *E. junaluska* held by the GSMNP in the model in the following manner: if two or more *E. junaluska* collections were reported from a stream, this was included as a confirmed presence for an eDNA sample collected from that site. This resulted in the inclusion of eight confirmed presence entries into the model. The analysis, which included the three PCR replicates used in the study, included 2,000 burn-in iterations, 2,000 iterations, one chain, and 20 thinned iterations. As suggested by the model authors (Diana et al. 2021), we used the default settings of the posterior means for all other model parameters. The code and data used in the model are available at <https://github.com/BenBrammell/E.-junaluska-eDNA-project>.

Historical data comparison.—We submitted a request for *E. junaluska* collection data from the GSMNP and obtained all records of *E. junaluska* sightings in the park (48 records). We compared these records to our eDNA sample collections and identified collocated sites. We considered a site collocated if the eDNA sample collection point and any sighting(s) were 8 km or less stream distance from one another.

RESULTS

Cytb sequencing.—The *E. junaluska* partial cytb sequence obtained in this study was 95.8% similar to the only other previously published *E. junaluska* cytb sequence. Similarity between this previously published sequence, the four *E. wilderae* sequences obtained in this study (92.1–90.8%), and the *E. junaluska* sequence obtained in this study are found in Supplemental Information Table S2.

In silico specificity testing.—The *E. junaluska* oligos (F + R primer and probe) developed in this study had assignment probabilities less than the recommend threshold of 0.30 (Kronenberger et al. 2022) with each species tested from genera other than *Eurycea* (0.10–0.21; Appendix Table). Assignment probabilities for the seven non-target *Eurycea* specimens (six species) tested ranged between 0.27 and 0.53 and only *E. lucifuga* (0.27) did not meet or exceed the 0.30 threshold (Appendix Table). Additionally, all oligos had a minimum collective six

mismatches with each sympatric salamander species considered (Appendix Table), and each individual oligo had at least one mismatch against all non-targets (Appendix Table). Each of our assay oligos had no mismatches and an assignment probability of 0.97 with both of the *E. junaluska* cytb sequences (Appendix Table).

In vitro specificity testing.—End-point reactions using *E. junaluska* forward and reverse primers with genomic tissue-extracted DNA from eight sympatric species, including most *Eurycea* species found in the GSMNP (the exception was *E. guttolineata*), revealed binding only for *E. lucifuga* and *E. wilderae* (Supplemental Information Figs. S2 and S3). qPCR testing using DNA from plasmid clones and gBlocks from these same species, however, revealed no amplification with any non-target species at DNA concentrations of both 10,000 and 100,000 copies per reaction (Supplemental Information Table S4). Mean Ct values for *E. junaluska* DNA reactions using gBlocks and plasmid-cloned DNA were 28.5 (gBlocks) and 30 (plasmid clone) for the 10,000 copies per reaction concentration and 25.0 (gBlocks) and 25.6 (clone) for the 100,000 copies per reaction concentration (Supplemental Information Table S4). Additional qPCR testing with tissue or swab-extracted *E. wilderae* DNA revealed no amplification for *E. wilderae* specimens collected from within or adjacent to the GSMNP; however, amplification occurred at high Ct values for an *E. wilderae* specimen collected in Georgia, USA (Table 2).

Assay validation and inhibition testing.—The LOD and LOQ values obtained from the curve-fitting model approach described by Klymus et al. (2020) were 2.7 and 8 copies per reaction, respectively, and the coefficient of determination (r^2 -value) for the curve was 0.99 with an amplification efficiency of 96.7% (Supplemental Information Table S5). Five water samples (Sample #38, #39, #44, #45, and #46) demonstrated inhibition and were treated to remove inhibitors. Each sample was retested and did not show inhibition in post-treatment testing.

Field survey results.—Four water samples were positive for *E. junaluska* DNA (Table 3); two samples amplified in one of three qPCR replicates, one amplified in two of three, and one amplified in three of three. Specific locality data has been withheld at the request of the GSMNP, but these samples were collected from the Abrams and Little River

TABLE 4. Number of sample collection sites listed by stream order according to the Strahler method of stream classification. The symbol – indicates a lack of an assigned order (two sample sites) and signifies that water samples were taken from seeps or temporary streams that did not appear on maps.

Stream Order	Number Sampled
6	1
5	9
4	14
3	9
2	9
1	6
–	2

drainages of the GSMNP (Table 3). All positive sites were in 4th order or higher streams (Table 4). No amplification was observed in any of the field blanks or PCR negative controls.

Amplicon sequencing.—Amplicons produced from environmental samples were sequenced, as recommended (Bockrath et al. 2022), to evaluate the possibility of false positives. Amplicons were sequenced bidirectionally and therefore provided complete coverage of the entire sequence. Sequenced amplicons were each 100% matches to the cytb sequence from an *E. junaluska* individual collected in Blount County, Tennessee (GenBank Accession # KF562550.1) and were also each 98.8% similar to the cytb sequence from an *E. junaluska* individual collected in Graham County, North Carolina (GenBank Accession # PP277535.1; Supplemental Information Table S6).

Occupancy modeling.—The estimated posterior mean probability of target species presence (*E. junaluska*) presence for all sites was 0.174 (95% CRI 0.0456–0.397; Table 5). The probability of collecting target eDNA in a water sample (θ_{11}) and the probability that a qPCR replicate from a sample containing target species eDNA would be positive (p_{11}) were 0.670 (95% CRI 0.181–0.999) and 0.471 (95% CRI 0.161–0.872; Table 5) respectively. Likewise, the probability of false positives was lower than model-expected values for both field 0.0118 (95% CRI 0.000101–0.0633) and lab 0.00406 (95% CRI 0.0000462–0.0201; Table 5) respectively. The analysis of conditional probability of species absence indicated that there was a low probability of sites being unoccupied when one or more qPCR replicate amplified (1 qPCR replicate = 0.095%, 2 = 0.032%, and 3 = 0.30%). All included covariates were found

TABLE 5. Occurrence probability of the Junaluska Salamander (*Eurycea junaluska*): observed and expected values of true and false positive probabilities. From the model of Griffin et al. (2020), which accounts for false positive and false negative errors in both sample collection and qPCR analysis in single-species assay eDNA studies.

Parameter	2.5 credible interval	Mean	97.5 credible interval	Expected value
Probability site is occupied by target species (ψ)	0.0454	0.169	0.392	0.5
Probability of eDNA collection in an occupied site (θ_{11})	0.205	0.678	0.999	0.9
Probability of failure to collect eDNA in an occupied site ($1-\theta_{11}$)	—	0.322	—	—
Probability of eDNA collection in an unoccupied site (θ_{10})	0.000062	0.012	0.0663	0.1
Probability of a positive qPCR replicate in a sample from an occupied site (p_{11})	0.175	0.459	0.832	0.9
Probability of a negative qPCR replicate in a sample from an occupied site ($1-p_{11}$)	—	0.541	—	—
Probability of a positive qPCR replicate in a sample from an unoccupied site (p_{10})	0.0000443	0.004	0.0169	0.1

to have Posterior Inclusion Probability (PIP) values > 0.5 for occupancy (east/west = 0.73, elevation = 0.63 m, and stream order = 0.778), suggesting their importance as predictors for *E. junaluska* occupancy (Supplemental Information Fig. S4).

Historical data comparison.—We submitted an official request and were granted access to all *E. junaluska* records held in the GSMNP database: 48 *E. junaluska* sightings, with coordinates, observed between 1976 and 2001 (Table 6). Specific locality information has been withheld in compliance with GSMNP requirements. Comparison of these field observation sites with our eDNA collection sites yielded 10 eDNA sample collection sites that met our collocation criteria. Of these, five had two or more *E. junaluska* observations. We observed eDNA detections in two of these five collocated sites: the two with the highest number (13 and 11) of *E. junaluska* observations (Table 6). We did not obtain eDNA detections for the remaining sites with more than two observations or for the two sites with a single observation (Table 6).

DISCUSSION

Eurycea junaluska is widely reported to be found in large versus small streams, consistent with the results of the present study in which each of the *E. junaluska* eDNA detections occurred in relatively large, high-order, low-elevation streams. Ideal

habitat for *E. junaluska* reportedly consists of quiet backwater areas of relatively large streams (Ryan and Sever 2005) with low-gradients ($< 2\%$) and abundant silt strata (Chattin et al. 2007). Dodd (2004) reported *E. junaluska* to occur in larger streams at elevations of ≤ 524 m, particularly on the Tennessee side of the GSMNP (Dodd 2003), including streams in which *E. junaluska* eDNA was detected in this study. In a leaf litter bag study within the GSMNP, Waldron et al. (2003) report observations of *E. junaluska* in three of five larger streams sampled but none in the smaller streams included in their study. Additionally, both Dodd (2003) and Ryan and Sever (2005) report *E. junaluska* to be more abundant on the western portion of the GSMNP in Tennessee relative to the eastern portion in North Carolina. Chattin et al. (2007) speculate that construction of a dam on the Little Tennessee River (forming the eastern border of the GSMNP in North Carolina) restricted *E. junaluska* to low-gradient streams adjacent to the impounded river channel. In a manner likewise consistent with these observations, each of our eDNA detections of *E. junaluska* occurred on the western side of the GSMNP within Tennessee.

We recognize both the importance of considering genetic data from sympatric species in eDNA surveys (Wilcox et al. 2014; Ogata et al. 2022) and the challenges presented by intraspecific genetic variation (Serrao et al. 2021; Brewer et al. 2025); thus, we have attempted to address each thoroughly in our approach. Of the *Eurycea* species

TABLE 6. Historical data (courtesy of the GRSMNP) regarding the Junaluska Salamander (*Eurycea junaluska*) sightings in the Great Smoky Mountains National Park, USA, between 1976 and 2001 (all data available for this species) and eDNA results for collocated sites. Column labels are Stream = lotic system of interest, Elevation = elevation in meters, Order = Strahler stream order, # of obs. = number of recorded observations in specified system, Larvae, Adults, and NS = observations noted for *E. junaluska* larvae, adults, or not specified, Years = year ranges in which observations were recorded, eDNA dist. = distance of *E. junaluska* eDNA sample collection point from its associated sighting report(s) with U indicating the eDNA site was upstream and D indicating the eDNA site was downstream of the sighting, and eDNA det. = *E. junaluska* was detected in X/X water samples collected in that system. In the two rightmost columns, the symbol – indicates that no eDNA sample was collected in that system.

Stream	Elevation (m)	Order	# of obs.	Larvae	Adults	NS	Years	eDNA dist. (km)	eDNA det.
1	338–521	5	13	3	–	10	1998–2001	< 0.2 D, < 0.2U	2/2
2	336–564	5/6	11	7	2	2	1998–1999	1.5 D, 2.4 U	2/3
3	417	6	6	1	–	5	1998–2000	< 0.2 U	0/1
4	442	4	4	2	–	2	1976–1998	< 0.2 D	0/1
5	529	5	2	–	–	2	2000	5.0 D	0/1
6	407	4	1	–	–	1	2000	< 0.2 D	0/1
7	474	2	1	1	–	–	1998	1.5 U	0/1
8	276–298	3	4	1	–	3	1999–2000	–	–
9	1121	2	1	1	–	–	1998	–	–
10	338	1	1	–	1	–	1998	–	–
11	389	4	1	–	–	1	2001	–	–
12	360	2	1	–	–	1	2000	–	–
13	775	1	1	–	–	1	2000	–	–
14	453	4	1	–	–	1	2000	–	–

sympatric with *E. junaluska*, *E. wilderae* is most closely related (Edgington and Taylor 2019), an observation reflected in our assay testing both *in silico* and *in vitro*. Studies using allozymes (Jacobs 1987), mitochondrial DNA (Kozak et al. 2006), and nuclear DNA (Pierson et al. 2023) are congruous that *E. wilderae* is not monophyletic but consists of three clades; only the southernmost of these (Clade M) occurs in the GSMNP and is sympatric with *E. junaluska* (Kozak et al. 2006; Pierson et al. 2023). Testing with four members of Clade M *in silico* and three *in vitro* reveals that our *E. junaluska* forward and reverse primers do recognize *E. wilderae* DNA during *in vitro* (1.0 µg/ml) end-point PCR tests but not in qPCR *in vitro* tests, even at concentrations far above those found in the environment (100,000 copies per reaction). Probe-based qPCR introduces an additional level of specificity into an assay, requiring that a third oligonucleotide (the probe) bind to a target sequence (Nagy et al. 2017). Regarding intraspecific variation, we note the 4.2% variation observed between the *E. junaluska* cytb sequence from the western portion (Blount County, Tennessee, GenBank # KF562550.1) and the sequence from the eastern portion (Graham County, North Carolina, GenBank # PP277535.1) of *E. junaluska*'s range.

Previous studies have reported cytb intraspecific variation to typically be < 1–2% (Shen et al. 2013; Lalitha and Chandavar 2018), seemingly making this intraspecific variation to appear relatively high. Our assay was designed based on conserved regions between the two sequences from opposite ends of the *E. junaluska* range, likely incorporating the extent of polymorphisms present in this species.

Eurycea junaluska is one of six named taxa of the *E. bislineata* species complex, a group that last shared a common ancestor in the Miocene (Bonett et al. 2014; Wray and Steppan 2017) and has long presented a challenge to taxonomists (Pierson et al. 2023) as a result of a lack of agreement concerning various datasets, including morphology (Sever 1989), allozymes (Jacobs 1987), and nuclear (Pierson et al. 2019; Stuart et al. 2020) and mitochondrial DNA (Kozak et al. 2006). Recent works have suggested that *E. junaluska* may more properly be categorized as a sub-taxa of *E. aquatica* (Pierson et al. 2023). Indeed, previous studies have noted similarities in behavior of *E. aquatica* and *E. junaluska*, such as migrating to breeding sites from unknown terrestrial retreats, which are not shared by other members of the *E. bislineata* complex (Graham et al. 2013). Our assay would likely recognize the one *E. aquatica*

cytb sequence available in GenBank (Accession # KF562543.1; six mismatches, AP = 0.44); however, given that the assay is intended solely to recognize *E. junaluska*, and because *E. aquaticus* occurs entirely allopatrically (Stuart et al. 2020), we believe this should not present an issue.

Assay sensitivities reported here (LOD = 2.7, LOQ = 8) are similar to or lower than those reported by other eDNA studies. Bell et al. (2022) reported values of 2 (LOD) and 20 (LOQ) copies per reaction for assays detecting *E. cirrigera* and *D. fuscus*. Kaganer et al. (2022) reported 10 copies per reaction as both the LOD and LOQ for an assay targeting Four-toed Salamanders (*Hemidactylium scutatum*). An LOD of 23 and LOQ of 75 copies per reaction were reported for an assay detecting the California Tiger Salamander (*Ambystoma californiense*, Kieran et al. 2020), while LOD values for assays detecting species of native and invasive crayfish in Europe were reported as one copy per reaction for the European Crayfish (*Astacus astacus*), the White-clawed Crayfish (*Austropotamobius pallipes*), the Stone Crayfish (*Austropotamobius torrentium*), and the Red Swamp Crayfish (*Procambarus clarkii*), 100 copies per reaction for the Signal Crayfish (*Pacifastacus leniusculus*), and 1,000 copies per reaction for the Narrow-clawed Crayfish (*Astacus leptodactylus*; King et al. 2022). The sensitivity of the assay presented here appears consistent with current eDNA single-species assay standards and is capable of detecting eDNA at low concentrations.

In the modeling approach we used to estimate occupancy and the probability of false positive and false negative errors, the results suggest a low probability of false positive errors but a somewhat higher probability of false negative errors. Water sample and qPCR replicate false positive probabilities were substantially lower than water sample and qPCR replicate true positive probabilities. Likewise, the probability of a false positive given only a single positive qPCR replicate was relatively low (10%) and decreased as positive qPCR replicates increased. These observations suggest that the *E. junaluska* qPCR eDNA detections observed in our study were likely true detections; however, both the probability of a true positive field sample (0.678) and the probability of a true positive lab sample (0.459) were lower than model expectations, suggesting moderate probabilities of false negative errors (field = 0.32 and lab = 0.54). Although these false negative error probabilities are less than some similar eDNA studies, such as Lyman et al. (2022), who reported

false negative probabilities of 0.73 (sample) and 0.67 (qPCR replicate) in eDNA surveys of an endangered rodent, both the sample and the qPCR replicate false negative error probabilities are higher than those reported in other eDNA studies. Kalogianni et al. (2023) reported sample (0.10 – 0.13) and qPCR replicate (0.15 – 0.16) probabilities in eDNA surveys of two rare *Barbus* (fish) species while Greenhalgh et al. (2024) reported false negative probabilities of 0.015 and 0.39 (sample) and 0.007 and 0.41 (qPCR replicate) in eDNA studies detecting two invasive crayfish species in Norfolk (UK), suggesting that improvement is possible in the level of both field and laboratory replication conducted in the present study. Additionally, although some eDNA studies report probabilities of eDNA collection in a sample (θ_{11}) that are similar to or exceed detection in a qPCR replicate (p_{11} ; Buxton et al. 2022; Kalogianni et al. 2023; Greenhalgh et al. 2024), a number of other studies report higher probability of detection in a qPCR replicate relative to sample collection (Lyman et al. 2022, Suebsuya et al. 2025). In our study, target organism eDNA concentration was low, approaching limits of detection. Although both greater numbers of field sample replicates and qPCR replicates have been demonstrated to reduce false negative errors (Cantera et al. 2019), the relatively low probability of detection in a qPCR replicate observed in the modeling conducted for the present study suggests an increased number of PCR replicates may enhance target species detection, as observed in previous studies targeting low-concentration eDNA (Hunter et al. 2017).

Numerous studies report *E. junaluska* to be exceptionally rare, even in areas where it occurs in highest abundance within its restricted range, and therefore difficult to study (Sever 1984; Ryan 1998; Ryan and Sever 2005). The type specimens for the original description were collected over a 3-y period (Sever et al. 1976), and 10 y after the collection of the first specimens, Sever (1983) reported having collected fewer than 50 metamorphosed individuals. Ryan (1998) reported never finding more than two adult *E. junaluska* at any sample site following a long-term study at three sites at which they are known to be most abundant. A survey for *E. junaluska* larvae in streams in Graham County, North Carolina revealed larvae or adults at only three of 63 sites after over 400 h of collecting (Ryan 1997), while in the same study, no adults or larvae were observed in two creeks previously known to contain *E. junaluska* (Ryan 1997). Our results appear to support these

observations: we detected *E. junaluska* DNA only in ideal habitat of areas previously known to harbor the largest, and perhaps most stable, *E. junaluska* populations. Certainly, false negatives are possible in any eDNA study, and although a positive eDNA detection provides compelling evidence of the presence of target DNA, the absence of detection can never be interpreted as the definite lack of the organism (Bockrath et al. 2022). For example, in lotic systems, eDNA of the Idaho Giant Salamander (*D. aterrimus*) was detected at 5 m downstream of an individual but not 50 m downstream (Pilliod et al. 2014). Additionally, eDNA concentrations have been widely established to correlate with biomass (Robinson et al. 2019; Guivas and Brammell 2020; Brys et al. 2021), including in an amphibian study that detected three species of troglobitic *Eurycea* (Adcock et al. 2023) in lotic systems in central Texas, USA and in a study detecting Northern Dusky Salamanders (*D. fuscus*) in lotic systems in eastern Canada (Plante et al. 2021). In our case, we searched for a small (SVL 37–49 mm; Ryan and Sever 2005) organism in lotic systems of varying size. Presumably, the probability of detection should have been much greater in smaller systems with less dilution, consistent with previous observations of increased dilution of eDNA in systems with greater rates of discharge (Van Driessche et al. 2023), perhaps highlighting both the significance of detection in the larger streams and lack thereof in the many smaller streams sampled in this study. Overall, our results support previous findings: *E. junaluska* is a rare salamander found primarily in large, low-elevation and low-gradient streams, primarily on the western side of the GSMNP.

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APPENDIX

APPENDIX TABLE. Mismatches and amplification probabilities for the Junaluska Salamander (*Eurycea junaluska*) oligos and salamander species of the Great Smoky Mountains National Park (GSMNP), USA. All salamander species reported in the GSMNP (Dodd 2003) are included along with the newly described Cherokee Black-bellied Salamander (*Desmognathus gvnigeusgwotli*) for which no sequence is currently available. Two species found nearby but not in the GSMNP, the Brownback Salamander (*Eurycea aquatica*) and Southern Two-lined Salamander (*Eurycea cirrigera*), are also included. Abbreviations are F = forward primer, R = reverse primer, P = probe (numbers below these columns represent the number of mismatches with the respective oligo), AP = assignment probability of the respective species with our assay as determined by machine learning model (Kronenberger et al. 2022), Seq. Accession # = the GenBank Accession number, and Len. (bp) = the length of the sequence in base pairs.

	Family	Species	Common name	AP	F	R	P	Seq. Accession #	Len. (bp)
1		<i>Ambystoma maculatum</i>	Spotted Salamander	0.10	6	6	4	EF036637.1	744
2	Ambystomatidae	<i>Ambystoma opacum</i>	Marbled Salamander	0.13	3	7	4	KT780868.1	720
3		<i>Ambystoma talpoideum</i>	Mole Salamander	0.11	5	6	5	EF036640.1	744
4	Cryptobranchidae	<i>Cryptobranchus alleganiensis</i>	Hellbender	0.11	5	3	7	AY691719.1	783
5		<i>Aneides aeneus</i>	Green Salamander	0.10	5	3	4	OM111015.1	365
6		<i>Desmognathus aeneus</i>	Seepage Salamander	0.18	4	8	3	EU314341.1	519
7		<i>Desmognathus conanti</i>	Spotted Dusky Salamander	0.14	7	5	4	EU314275.1	519
8		<i>Desmognathus imitator</i>	Imitator Salamander	0.14	8	7	6	EU314336.1	519
9		<i>Desmognathus marmoratus</i>	Shovel-nosed Salamander	0.15	6	5	4	EU314355.1	519
10		<i>Desmognathus monticola</i>	Seal Salamander	0.10	6	7	5	AY691738.1	783
11		<i>Desmognathus ocoee</i>	Ocoee Salamander	0.17	7	6	4	EU314297.1	519
12		<i>Desmognathus quadramaculatus</i>	Black-bellied Salamander	0.16	7	6	4	EU314374.1	519
13		<i>Desmognathus santeetlah</i>	Santeetlah Salamander	0.14	8	7	6	EU314334.1	519
14		<i>Desmognathus gvnigeusgwotli</i>	Cherokee Black-bellied Salamander	—	—	—	—	N/A	—
15		<i>Desmognathus wrighti</i>	Pygmy Salamander	0.10	10	7	6	EU314340.1	519
-		<i>Eurycea aquatica</i>	Brownback Salamander	0.44	3	2	1	KF562543	674
-		<i>Eurycea cirrigera</i>	Southern Two-lined Salamander	0.53	4	2	1	MZ485475	380
16	Plethodontidae	<i>Eurycea guttolineata</i>	Three-lined Salamander	0.42	4	4	1	JQ920625.1	1,012
17		<i>Eurycea junaluska</i>	Junaluska Salamander	0.97	0	0	0	KF562550.1	674

APPENDIX TABLE, CONTINUED

	Family	Species	Common name	AP	F	R	P	Seq. Accession #	Len. (bp)
-		<i>Eurycea junaluska</i>	Junaluska Salamander	0.97	0	0	0	PP277535.1	635
18		<i>Eurycea longicauda</i>	Long-tailed Salamander	0.51	5	3	1	JQ920624.1	1,012
19		<i>Eurycea lucifuga</i>	Cave Salamander	0.27	3	2	5	EF044248.1	982
20		<i>Eurycea wilderae</i>	Blue Ridge Two-lined Salamander	0.39	3	1	2	PP393049	694
-		<i>Eurycea wilderae</i>	Blue Ridge Two-lined Salamander	0.30	3	2	3	PP393050	702
21		<i>Gyrinophilus porphyriticus</i>	Spring Salamander	0.16	6	2	5	EU336391.1	783
22		<i>Hemidactylium scutatum</i>	Four-toed Salamander	0.19	4	6	4	AY691752.1	783
23		<i>Plethodon glutinosus</i>	Northern Slimy Salamander	0.09	6	7	8	MN723529.1	1,117
24		<i>Plethodon jordani</i>	Jordan's Salamander	0.10	5	8	6	DQ994947.1	649
25		<i>Plethodon metcalfi</i>	Southern Gray-cheeked Salamander	0.08	8	8	5	DQ994957.1	632
26		<i>Plethodon oconaluftee</i>	Southern Appalachian Salamander	0.10	6	7	5	DQ994966.1	649
27		<i>Plethodon serratus</i>	Southern Red-backed Salamander	0.16	4	8	6	KM225293.1	1,140
28		<i>Plethodon ventralis</i>	Southern Zigzag Salamander	0.14	5	4	5	DQ994994.1	631
29		<i>Pseudotriton montanus</i>	Mud Salamander	0.16	3	3	4	KF562586.1	674
30		<i>Pseudotriton ruber</i>	Red Salamander	0.18	5	2	4	AY528404.1	1,118
31	Proteidae	<i>Necturus maculosus</i>	Common Mudpuppy	0.21	7	4	3	AY691724.1	783
32	Salamandridae	<i>Notophthalmus viridescens</i>	Eastern Newt	0.14	8	3	4	AY691731.1	783